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Biomarkers and genetic modulators of cerebral vasculopathy in sub-Saharan ancestry children with sickle cell anemia

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ABSTRACT

We investigated biomarkers and genetic modulators of the cerebral vasculopathy (CV) subphenotype in pediatric sickle cell anemia (SCA) patients of sub-Saharan African ancestry. We found that one *VCAM1* promoter haplotype (haplotype 7) and *VCAM1* single nucleotide variant rs1409419_T were associated with stroke events, stroke risk, as measured by time-averaged mean of maximum velocity in the middle cerebral artery, and with high serum levels of the hemolysis biomarker lactate dehydrogenase. Furthermore, VCAM-1 ligand coding gene *ITGA4* variants rs113276800_A and rs3770138_T showed a positive association with stroke events. An additional positive relationship between a genetic variant and stroke risk was observed for *ENPP1* rs1044498_A. Conversely, *NOS3* variants were negatively associated with silent cerebral infarct events (VNTR 4b_allele and haplotype V) and CV globally (haplotype VII). The -alpha$^{3.7kb}$–thal deletion did not show association with CV. However, it was associated with higher red blood cell and neutrophil counts, and lower mean corpuscular volume, mean corpuscular hemoglobin and red cell distribution width.

Our results underline the importance of genetic modulators of the CV sub-phenotype and their potential as SCA therapeutic targets. We also propose that a biomarker panel comprising biochemical, hematological, imaging and genetic data would be instrumental for CV prediction, and prevention.

1. Introduction

Sickle cell anemia (SCA), the homozygous form of the c:20A > T mutation in the beta-globin gene, is the most common and severe presentation of sickle cell disease (SCD). High birth rates and child mortality are most frequent in sub-Saharan Africa, however, population movements are leading to a wider distribution, emphasizing the global health emergence status of the disease [1–3]. In children, the most common sub-phenotypes are cerebral vasculopathy (CV), acute chest syndrome, hyposplenism, renal disease and painful crises. CV is a major complication and comprises overt stroke, silent cerebral infarcts (SCIs), transient ischemic attacks and frequently neurocognitive complications

Abbreviations: SCA, sickle cell anemia; SCD, sickle cell disease; CV, cerebral vasculopathy; SCI, silent cerebral infarction(s); LDH, lactate dehydrogenase; TAMMV, time-averaged mean velocity in the middle cerebral artery; TCD, transcranial Doppler ultrasound; MRI, magnetic resonance imaging; MRA, magnetic resonance angiography; HC, hydroxycarbamide; HbS, hemoglobin S; HbF, fetal hemoglobin; Hb, total hemoglobin; RBC, red blood cells; WBC, white blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red cell distribution width; NGS, next-generation sequencing; VCAM-1, vascular cell adhesion molecule 1; ITGA4, very-late antigen 4; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; TFBS, transcription factor binding site(s)

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at a later stage. Children with SCA have a much higher risk of stroke than the general pediatric population. The prevalence of overt stroke approaches 11% by age 20 years. On the other hand, SCIs have been found in up to 37% of children with SCA [4].

The current standard of care for stroke prevention in SCA children is Transcranial Doppler (TCD) screening – through measurement of time-averaged mean velocity (TAMMV) in the middle cerebral artery – followed by regular blood transfusions therapy and hydroxycarbamide (HC) treatment. Despite its high sensitivity, TCD still does not allow identification of all SCA children that will experience a stroke and, conversely, children with high TAMMV (> 200 cm/s) may not develop stroke [5]. Moreover, blood transfusion/HC therapies are not without limitations or adverse side effects [6]. On the other hand, although diagnosis with magnetic resonance imaging, MRI, (or angiography, MRA) is recommended for early diagnosis of SCIs and recognition of large vessel stenosis, MRI/MRA are not useful to identify patients at risk of developing SCIs or large vessel stenosis [7]. A more specific and sensitive panel of biomarkers for CV prediction and prognosis, that includes genetic variants with disease modifying effects, is therefore of the utmost importance.

In previous studies, we identified variants in *VCAM1*, *NOS3* and *HBA* with a positive association with chronic hemolysis, a known pathophysiological SCA mechanism [8]. Building on those results we aimed, in this work, to assess if those variants were also associated with pediatric CV in a sub-Saharan SCA population. Our candidate gene approach also focused on the VCAM-1 ligand gene, *ITGA4*, and for comparison purposes, we included the three genetic variants (*PON1* rs662, *ENPP1* rs1044498 and *GOLGB1* rs3732410) previously reported in association with pediatric stroke in SCA patients [7,9,10].

2. Materials and methods

2.1. Ethical statement

Ethical approval for the study was granted by the institutional review boards of Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA) and of participant hospitals, in line with the principles of the Declaration of Helsinki. The aim and study procedures were explained to the children's parents (or legal guardians) and they provided informed written consent prior to their enrolment in this study.

2.2. Study population

This case-control study was performed at INSA in cooperation with four hospitals in the Greater Lisbon area – Hospital D. Estefânia, Hospital de Santa Maria, Hospital Fernando Fonseca and Hospital Garcia de Orta, the four largest centers of that metropolitan area. These centers combined receive the highest numbers of SCA pediatric patients in our country. Seventy unrelated pediatric patients (≥3 years old) of direct sub-Saharan African ancestry diagnosed with SCA were selected. Exclusion criteria included age < 3 years old, non-African ancestry, previous HC treatment or having received a blood transfusion in the 120 days prior to enrolment.

Data obtained from patients and parents (or legal guardians) interviews, which included demographic characteristics (age, gender, parents' geographic origin), were collected. Hemoglobin profiles (HbS, HbF), hematological parameters [RBC, leukocyte, neutrophil and platelet counts, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW)] as well as hemolysis biochemical and hematological biomarkers [serum LDH and total bilirubin and reticulocyte count] were retrospectively collected from patients' hospital records. All these parameters were obtained by standard procedures and HbF levels, in particular, were measured by high-performance liquid chromatography (HPLC). The data collected for each parameter were the result of, at least, three different time-point measurements, performed in steady-state periods, and prior to any treatment with HC and more than > 120 days after receiving a blood transfusion. The patients were analyzed according to CV outcome, or stroke risk, in the following groups (i) overt ischemic stroke (≥1 episode), henceforth designated as “stroke” (n = 62); (ii) “silent cerebral infarct”(≥1 event), SCI (n = 53); (iii) stroke and/or SCI, as confirmed by MRI or MRA, (n = 62); or “stroke risk” (n = 60), with risk stratification provided by the TAMMV values, obtained during TCD, as follows: (a) high risk – TAMMV ≥200 cm/s; (b) conditional (or moderate) risk – 200 cm/s > TAMMV ≥170 cm/s; and (c) low risk – TAMMV < 170 cm/s.

2.3. Genotyping

Genomic DNA was isolated from peripheral blood samples of each patient using the MagNA Pure LC Instrument (Roche Diagnostics GmbH, Mannheim, Germany). All samples were anonymized and specific genotypes could be linked to phenotypes only through the main study database.

The homozygous status for the SCA mutation in the *HBB* gene (c.20A > T) was confirmed by polymerase chain reaction followed by restriction fragment length analysis (PCR-RFLP) with the endonuclease *Bsu*36 I. Beta-globin cluster haplotypes were assigned after examining six restriction endonucleases sites within the cluster: *Xmn* I (5′ to *HBG2*), *Hin*d III (within the *HBG2* and *HBG1*) *Hin*c II (within and 3′ to *ψHBB*) and *Hin*f I (3′ to *HBB*). Aliquots of the amplified products were digested with the appropriate enzymes under the conditions recommended by the manufacturers. Haplotypes were determined by the presence or absence of cleavage at each site and by analyzing the compiled pattern through comparison to known patterns [11]. The -$\alpha^{3.7kb}$–thal deletion was assessed by gap-PCR [12].

Putative modifier genes were identified through previous studies by our group [8] and from other published reports based on the influence on the phenotypes of interest. These candidate genes were used to identify and genotype SNPs and other variants in patient samples. For *VCAM1*, *NOS3*, *PON1*, *ENPP1* and *GOLGB1* genes genotyping was performed using TaqMan-based PCR with commercially available or customized primers.

2.3.1. Screening for ITGA4 variants by next-generation sequencing (NGS)

In order to search for variants in the regulatory region of *ITGA4* gene, NGS analysis was used on a long PCR fragment (4.1 kb), including its flanking regions. PCR was performed using the primers FW5’-CAG AGGCTCATTAGGACCC-3′ and Rv5′-CCTTGCGGTACTATCCAGGC-3′ and the FailSafe enzyme with the PreMix G (Epicentre, Illumina, San Diego).

The NGS workflow consisted in five steps: PCR product purification using paramagnetic beads (Agentcourt, Ampure XP); double-stranded DNA quantification in a Qubit fluorometer; DNA library preparation using the Nextera XT kit (Illumina, San Diego) following the manufacturer's instructions; high throughput sequencing in a MiSeq benchtop sequencer (Illumina, San Diego); data analyses were performed using the following tools: Sequencing Analysis Viewer (v1.8.46, Illumina) and FastQC (v0.11.5, Babraham Bioinformatics,https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) were used for quality analysis. The MiSeq® Reporter software package (v2.6.2, Illumina) was used for read mapping (with Burrows-Wheeler Aligner) and variant calling and filtering (with Genome Analysis Toolkit). FastQ screen (v.0.9.3, Babraham Bioinformatics) was used to screen for contamination between samples. Variant Effect Predictor (www.ensembl.org) was used to annotate variants and Integrative Genomics Viewer (v.2.3.86) (Broad Institute; [13]) was used for visualization of reads and variants. Validation of the variants was performed using automated Sanger sequencing, after amplification with customized primers in the 3130XI Genetic Analyser, (Applied Biosystems). The results were analyzed using FinchTV v.1.4.0 software (Geospiza, Inc.). The genotyping results were added to the previously created database.

2.3.2. Haplotype reconstruction

Haplotype reconstruction was performed using PHASE software v2.1 developed by Mathew Stephens at Washington University, according to the developer's instructions (https://els.comotion.uw.edu/express_license_technologies/phase). Haplotypes were reconstructed for genetic variants in *NOS3* (rs2070744, intron 4_27 bp VNTR, rs1799983), in the promoter of *VCAM1* (rs1409419, rs3917024, rs3917025, rs3783598, rs1041163, rs3783599) and for genetic variants of *ITGA4* (rs1375493, rs35723031, rs10562650; rs1839269 and rs1839268).

2.3.3. In silico analysis

Population allele and genotype frequencies were recorded for each observed variant using the Ensembl browser (www.ensembl.org). SNPs' sequences were retrieved using the NCBI SNP search engine (http://ncbi.nlm.gov/snp).

Transcription factor binding sites (TFBS) analysis was performed for the variants identified in the regulatory regions, using the MatInspector tool (Genomatix, Munich, Germany), to evaluate potential effects on the regulation of gene expression by altering putative TFBS. Only results above the 0.85 threshold were considered, which corresponds to a maximum of 15% dissimilarity between the identified sequence and the consensus sequence. A comparison with previously reported consensus sequences of TFBS for the *VCAM1* [14] and the *ITGA4* [15] promoters was performed. The sequences of the identified variants were not found to overlap with any of the previously reported TFBS consensus sequences. Hence, no strong effects for these genes' expression are to be expected as a result of the presence of those variants.

2.3.4. Statistical analysis

The analyses were performed using the SPSS software (version 25.0, IBM Inc., Chicago, USA). For descriptive analysis, continuous variables were represented as medians and interquartile ranges (IQR). To evaluate the Gaussian distribution of variables, Shapiro-Wilk normality tests where applied. We used the Mann-Whitney *U* test to compare the medians of variables. Categorical variables are represented as number, frequencies and percentages. The chi-square test or the Fisher's exact test were used to compare categorical variables on bivariate analysis. Statistical significance was defined as $p < 0.05$.

The minor allele for each variant was evaluated for potential association with stroke, SCI, cerebral vasculopathy (stroke and SCI combined) or risk (as measured by TCD-TAMMV values), *via* 2 × 2 phenotype × genotype contingency tables. Only polymorphisms with a minor allele frequency (MAF) > 5% were considered for association analysis. Phenotypes were classified as follows: (i) “stroke” (at least one previous overt ischemic stroke event, as confirmed by MRI/MRA) or “no stroke” (no clinically/imaging identified stroke event); (ii) “SCI” (at least one event as identified by MRI/MRA) or “no SCI” (no SCI events, as confirmed by MRI); (iii) “cerebral vasculopathy” (at least one overt ischemic stroke and/or SCI event) or “no cerebral vasculopathy”; and (iv) “risk of stroke” (high/moderate: TAMMV ≥ 200 cm/s or 199 ≥ TAMMV ≥ 170 cm/s; low: TAMMV < 170 cm/s).

Each variant was also evaluated for potential association with biochemical and hematological parameters, including hemolysis biomarkers (LDH, total bilirubin, reticulocyte count).

3. Results

3.1. Population description and genotyping

This study was performed on seventy unrelated SCA patients, (age range: 3–16 years, 40 males, 30 females), living in Portugal but of direct sub-Saharan ancestry, with parental geographic origin mainly from Angola, São Tomé and Príncipe and Cape Verde (Table 1).

Table 1
Demographic, neurological status and laboratory parameters of the population in this study.

Age (years)		3–16	
Gender		*n*	%
Male		40	57.1
Female		30	42.9
Parental geographic origin		*n*	%
Angola		42	60.0
São Tomé and Príncipe Islands		8	11.4
Cape Verde		5	7.1
Guinea-Bissau		7	10.0
Guinea-Conakry		1	1.4
Nigeria		1	1.4
Double origin		6	8.6
Neurological status (n = 70)		*n*	%
Stroke	Yes	15	24.2
(n = 62)	No	47	75.8
SCI	Yes	9	16.9
(n = 53)	No	44	83.0
CV	Yes	24	38.7
(n = 62)	No	38	61.3
Stroke risk	High (TAMMV ≥ 200 cm/s)	25	41.7
(n = 60)	Moderate (170 cm/s ≤ TAMMV ≤ 200 cm/s)	6	10.0
	Low risk (TAMMV < 170 cm/s)	29	48.3
Hematological parameters		Median	IQR
Hb S (%)		79.9	14.5
Hb F (%)		10.7	11.7
Hb (g/dL)		8.0	1.3
RBC ($\times 10^{12}$/L)		3.0	0.7
MCV (fL)		81.3	14.3
MCH (pg)		26.9	6.0
Reticulocytes (%)		9.9	6.3
RDW (%)		21.2	4.5
Platelets ($\times 10^9$/L)		404.1	167.7
WBC ($\times 10^9$/L)		12.6	4.8
Neutrophils ($\times 10^9$/L)		5.6	2.65
Biochemical parameters		Median	IQR
LDH (U/L)		636.3	473.4
Total bilirubin (mg/dL)		2.6	1.9
HBB cluster haplotype		*n*	%
Bantu/Bantu		38	54.3
Senegal/Senegal		11	15.7
Benin/Benin		3	4.3
Compound heterozygous		17	24.2
Atypical		1	1.4

A total of seventy-one genetic variants were identified of which twenty-eight (MAF > 0.05) were used in the association studies – seven in *VCAM1*, five in *NOS3*, three in *GOLGB1*, one in *PON1*, one in *ENPP1*, one in *HBA* (-$\alpha^{3.7kb}$–thal) and ten in *ITGA4* (Appendix Table A.1). We were able to reconstruct 16 haplotypes, and used ten of them (frequency > 0.05) for statistical analysis. Concerning the *HBB* gene cluster haplotypes, only the more frequent genotypes, Bantu/Bantu and Senegal/Senegal, were used for statistical analysis (frequency > 0.05).

3.2. In silico analysis

In silico analysis of the *VCAM1* gene promoter variants was focused on those with MAF > 0.05, except rs3917025 due to its occurrence in only one haplotype (Haplotype 3). The rs1041163_C, rs1409419_T and rs3917025_delCT *VCAM1* alleles were classified (according to ClinVar and Ensembl's VEP and Mat Inspector) as intergenic variants with potential modifying impact, although with no major pathologic effects. Potential changes resulting from the presence of the rs1041163_C allele include (i) TFBS alteration for RXRF transcription factor, substituting it for a PRDF site, and (ii) loss of a FHXB TFBS. The presence of rs1409419_T would lead to a potential gain of binding sites, in particular, for EVI1, Oct1 and Barx2. Regarding rs3917025_delCT, a potential gain of a FAST1 (FoxH1) TFBS was indicated.

Table 2
Association of biochemical and hematological parameters of SCA patients with stroke and stroke risk.

Parameter (units)	n	Stroke		p
		Yes	No	
		Medians (IQR)	Medians (IQR)	
HbF (%)	64	3.2 (9.3)	11.9 (10.3)	0.018
MCH (pg)	70	21.2 (20.8)	27.4 (6.0)	0.005

Parameter (units)	n	Stroke risk		p
		High + moderate	Low	
		Medians (IQR)	Medians (IQR)	
HbF (%)	64	8.5 (10.2)	12.1 (10.7)	0.043
Platelets ($\times 10^9$/L)	61	442.0 (156.4)	363.2 (124.2)	0.017
Neutrophils ($\times 10^9$/L)	61	6.3 (2.5)	4.9 (2.6)	0.009
LDH (U/L)	65	761.5 (535.7)	510.0 (325.3)	< 0.001

Values indicated as medians (interquartile range in brackets); n - number of patients; HbF - hemoglobin F; MCH - mean corpuscular hemoglobin; LDH - lactate dehydrogenase.

3.3. Association of biochemical and hematological parameters with cerebral vasculopathy

We observed significant associations of both stroke and stroke risk with several biochemical and hematological parameters (Table 2). Lower HbF percentages and MCH values were positively associated with stroke, while stroke risk was associated not only to lower HbF percentage but also to higher levels of coagulation, inflammation and hemolysis markers (Table 2).

3.4. Association of genetic variants with biochemical and hematological parameters

Genetic variants analyzed in our study, using the dominant genetic test model, showed association with both hematological and biochemical parameters, whether individually or as part of specific haplotypes (Table 3). *VCAM1* rs1041163 (CC + TC), *VCAM1*_haplotype 3, *ITGA4* rs113276800 (CA) and *ITGA4* rs3770138 (TT + CT) showed an association with lower levels of HbS. Conversely, higher HbF percentages were observed in association with rs1041163 (CC + TC), *VCAM1*_haplotype 3 and with Senegal/Senegal haplotype.

Namely, *VCAM1* as well as *ITGA4* variants, individually or within a haplotype context, were significantly associated with traditional biomarkers of disease severity, such as lower HbS percentage and higher LDH and total bilirubin values.

As for *PON1* rs662, the AA and GA genotypes showed a positive association with high platelets counts characteristic of a pro-coagulant state. Regarding *GOLGB1*, no significant association was found between the presence of rs3742410_C and hematological or biochemical parameters. However, we found two other *GOLGB1* SNPs while analyzing rs3732410_C – rs61746571_G and rs33988592_A, a synonymous and a missense variant, respectively. The rs61746571_G seems to be in linkage disequilibrium with variant rs3732410_C. On the other hand, rs33988592 AA and GA genotypes showed an association with lower values of inflammation markers (Table 3).

Table 3
Genetic variants association with the hematological and biochemical parameters.

Gene	Variant	Allele change or haplotype	Parameter (unit; nr. patients)		p
			HbS (%; n = 60)		
			Var	*No var*	
VCAM1	rs1041163	T > C	74.5	83.5	0.019
	Haplotype 3	C/C/CT/T/C/C	73.7	81.4	0.034
	rs33988592	G > A	11.3	13.4	0.030
ITGA4	rs113276800	C > A	69.0	80.4	0.012
	rs3770138	C > T	66.1	80.7	0.003
			HbF (%; n = 64)		
			Var	*No var*	
VCAM1	rs1041163	T > C	13.0	7.0	0.014
	Haplotype 3	C/C/CT/T/C/C	14.5	9.3	0.005
HBB	Haplotype	Sen/Sen	13.6	9.2	0.038
			Hb (g/dL; n = 65)		
			Var	*No var*	
HBB	Haplotype	Sen/Sen	8.1	7.9	0.022
			RBC ($\times 10^{12}$/L; n = 64)		
HBA	$-\alpha^{3.7kb}$ del	$\alpha\alpha > -\alpha^{3.7kb}$	3.1	2.7	0.008
			MCV (fL; n = 69)		
NOS3	rs2070744	T > C	87.3	79.9	0.024
	Haplotype IV	T/4a/G	78.7	83.2	0.032
HBA	$-\alpha^{3.7kb}$ del	$\alpha\alpha > -\alpha^{3.7kb}$	75.7	85.7	< 0.001
HBB	Haplotype	Sen/Sen	90.1	80.6	0.039
			MCH (pg; n = 70)		
			Var	*No var*	
HBA	$-\alpha^{3.7kb}$ del	$\alpha\alpha > -\alpha^{3.7kb}$	25.1	29.0	0.001
			RDW (n = 70)		
			Var	*No var*	
NOS3	rs2070744	T > C	19.6	21.6	0.031
	Haplotype I	C/4a/G	19.6	21.5	0.044
HBA	$-\alpha^{3.7kb}$ del	$\alpha\alpha > -\alpha^{3.7kb}$ del	20.8	21.2	0.021
			Platelets ($\times 10^9$/L; n = 61)		
			Var	*No var*	
PON1	rs662	G > A	442.0	378.1	0.028
			WBC ($\times 10^9$/L; n = 67)		
GOLGB1	rs33988592	G > A	11.3	13.4	0.030
HBB	Haplotype	Sen/Sen	9.9	13.0	0.030
			Neutrophils ($\times 10^9$/L; n = 61)		
			Var	*No var*	
GOLGB1	rs33988592	G > A	5.0	6.2	0.013
HBA	$-\alpha^{3.7kb}$ del	$\alpha\alpha > -\alpha^{3.7kb}$	5.7	5.5	0.010
HBB	Haplotype	Bantu/Bantu	6.2	5.0	0.036
	Haplotype	Sen/Sen	4.7	6.2	0.014
			LDH (U/L; n = 65)		
			Var	*No var*	
VCAM1	rs1409419	C > T	748.0	517.0	< 0.001
	Haplotype 7	T/C/CT/T/T/C	748.0	517.0	< 0.001
ITGA4	Haplotype A	G/GA/TT	611.5	1269.0	0.003
			Bilirubin (mg/dL; n = 69)		
			Var	*No var*	
VCAM1	rs3783613	G > C	3.2	2.4	0.026

Var – presence of variant allele or haplotype; ***No var*** – absence of variant allele or haplotype; HbS – hemoglobin S; HbF – fetal hemoglobin; Hb – total hemoglobin; RBC – red blood cells; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; RDW – red cell distribution width; WBC – white blood cells; LDH – lactate dehydrogenase; Sen - Senegal.

Table 4
Genetic variants association with cerebral vasculopathy.

Gene	Variant	Stroke (n = 62)				
		Yes	No	p	OR	95% CI
VCAM1	rs1409419_TT + CT	12	23	0.041	4.17	1.04–16.73
	rs1409419_CC	3	24			
	Haplotype 7	12	23	0.041	4.17	1.04–16.73
	Haplotype X	3	24			
ITGA4	rs113276800_CA	4	3	0.025	7.62	1.39–41.65
	rs113276800_CC	7	40			
	rs3770138_TT + CT	4	4	0.045	5.57	1.12–27.67
	rs3770138_CC	7	39			

Gene	Variant	SCI (n = 53)				
		Yes	No	p	OR	95% CI
NOS3	VNTR_4b	6	42	0.030	0.10	0.01–0.69
	VNTR_4a + 4c	3	2			
	Haplotype V	4	36	0.031	0.18	0.04–0.81
	Haplotype Y	5	8			

Gene	Variant	CV (n = 62)				
		Yes	No	p	OR	95% CI
NOS3	Haplotype VII	1	23	0.006	0.08	0.01–0.69
	Haplotype Z	13	25			

Gene	Variant	Stroke risk (n = 60)				
		High + moderate	Low	p	OR	95% CI
VCAM1	rs1409419_TT + CT	23	11	0.009	4.71	1.57–14.13
	rs1409419_CC	8	18			
	Haplotype 7	23	11	0.009	4.71	1.57–14.13
	Haplotype X	8	18			
NOS3	Haplotype V	18	24	0.050	0.29	0.09–0.96
	Haplotype Y	13	5			
ENPP1	rs1044498_AA + CA	14	5	0.026	4.03	1.21–13.42
	rs1044498_CC	16	23			

Haplotype X – presence of any of the other *VCAM1* promoter haplotypes studied; Haplotype Y – presence of any of the other *NOS3* haplotypes studied; SCI – silent cerebral infarction; CV – cerebral vasculopathy; 95% CI – 95% confidence interval.

3.5. Association of genetic variants with cerebral vasculopathy and cerebral vasculopathy risk

We found a significant association between the presence of several of the variants identified and CV/CV risk (Table 4). Namely, *VCAM1* rs1409419 (TT + CT), haplotype 7, *ITGA4* promoter rs113276800 (CA) and rs3770138 (TT + CT), showed a positive association with stroke. *ITGA4* variant rs3770138 (TT + CT) was also positively associated with CV as a whole. Positive associations were also found between high TAMMV values and *VCAM1* rs1409419 (TT + CT), *VCAM1* promoter haplotype 7, and also with the *ENPP1* rs1044498_A allele. On the other hand, *NOS3* intron 4 VNTR_4b allele and haplotype V were negatively associated with SCI, while haplotype VII showed a negative association with CV overall.

4. Discussion

Our study aimed to assess demographic, clinical, biochemical, hematological, genotyping and imaging data to design a potential biomarker panel for CV prognosis in children with SCA. With this approach, we were able to identify statistically significant associations of biochemical, as well as hematological parameters, with genetic variants and CV.

In silico analysis of the *VCAM1* rs1409419_T allele indicated particularly interesting potential TFBS gain, namely for EVI1, Oct1 and Barx2 transcription factors. EVI1 is of special note due to its complexity, multiple targets and modulation of several numerous processes, including cell migration, adhesion and proliferation [16]. It may cooperate with FOS transcription factor to limit cell adhesion while enhancing cell proliferation [16]. Conversely, Oct1 is a TF known to promote a transcriptional repression/silencing effect, which would potentially result in *VCAM1* down-regulation. On the other hand, a Barx2 binding site gain, predicted as a result of the rs1409419_T presence, has been shown to promote murine muscle cell differentiation, by interacting with muscle regulatory factors, whereas its loss would lead to decreased adhesion properties [17]. Hence, it is reasonable to assume that a gain could result in increased adhesion properties. All the TFs which expression may be affected by the significantly associated variants are mainly involved in development and in different tissues following the proposed role of VCAM-1 [18]. It is important to emphasize that while we can address the potential effects of the individual *VCAM1* variants' genotypes, the most important modifying role on disease manifestation would probably arise in the context of the haplotypes that encompass them. The one exception seems to be rs1409419_T given the overlapping findings observed for this variant and haplotype 7, which includes it.

We found that variants in the gene that encodes the VCAM-1 ligand, ITGA4, namely rs113276800_A and rs3770138_T were also positively associated with stroke. Furthermore, the latter was associated with CV globally. Given its role in WBC, reticulocyte and sickle erythrocyte adhesion to the activated SCA endothelium, this finding further underlines the role of cell-endothelium adhesion in the severe CV subphenotype. *ITGA4* rs113276800 has been previously described in association with multiple sclerosis and, as in our case, no AA individuals were observed [19,20]. This variant is located in the *ITGA4* promoter region near the AP-2 binding sites and the AA genotype may, therefore, cause a negative expression of the integrin α4 subunit [21]. We also found that the *ITGA4* rs1375493, rs35723031 and rs10562650 variants behaved similarly in our group of patients, most of whom were heterozygous for the three of them simultaneously. Furthermore, the co-occurrence of minor alleles in haplotype A – which, to our knowledge, has not been previously described – was associated with lower LDH values and potentially to a protective effect against hemolysis.

NOS3 (or eNOS) is the major NO-producer enzyme in the cardiovascular system, playing a crucial role in vascular tone control, vascular remodeling and proliferation. Furthermore, in SCA, NO bioavailability plays a very important modulating role, primarily through scavenging by cell-free hemoglobin [22]. The rs2070744 variant, located at position −786 of the *NOS3* gene 5′ flanking region, has been correlated with cardiovascular disease, namely its C allele, although there is still debate about how it affects mRNA and protein levels. In our study, we did not find any association of this variant with CV or stroke risk, which may be in accordance with previous reports of no significant differences between CC and TT genotypes effect on *NOS3* promoter activity [23]. The fact that the rs2070744_C has different distributions in different ethnic backgrounds [24] may also be responsible, to some extent, for these differences, since that allele is more frequent in Caucasians and our study population is of sub-Saharan origin. However, we observed a significant association between CC and TC genotypes of this variant and lower RDW, which has been discussed as a possible biomarker of lower cerebrovascular disease risk [25]. Lower RDW values (or reduced anisocytosis) would potentially act as a protective factor in consonance with what we observed for *NOS3*_haplotype V, which includes allele C, and CV protection. Although rs2070744 has been described in association with cardiovascular disease [26], its role in ischemic stroke has not been consensual. *NOS3*_haplotype V also includes intron 4 VNTR_4b allele, a variant that showed a protective effect against SCI. However, no relationship with any of the CV presentations studied here was observed. On the other hand, the *NOS3* rs1799983_T allele, which leads to aspartate for glutamate substitution at eNOS position 298, has been previously reported to be related to deficient eNOS caveolar localization and deficient shear stress response leading to reduced enzymatic activity. This SNP has been found in some populations to be more prevalent in patients with coronary artery disease, ischemic stroke and arterial hypertension [27]. However, in our study population, we did not observe any relationship of rs1799983 TT or GT genotypes with CV, biochemical or hematological parameters.

Several studies have identified other candidate gene polymorphisms as potentially affecting the risk of CV. However, the results published so far have been conflicting. A GWAS study by Flanagan et al. [9], performed in a large cohort of mainly African American SCA pediatric patients, showed a decreased risk of clinically overt stroke in association with *GOLGB1* rs3732410_G (Y1212C) and *ENPP1* rs1044498_C (K173Q) mutations, whereas *PON1* rs662_C (Q192R) was associated with increased risk of stroke [9]). In the same study, *GOLGB1* Y1212C was associated with reduced TCD velocities and lower frequencies of SCIs. Conversely, reports from Martella et al. [7] and Belisário et al. [10] indicated a link between *ENPP1* rs1044498_A and increased stroke risk as well as high TCD velocities. In our study, the *ENPP1* rs1044498_A allele was found in 18% of patients compared to 68.33% of Martella et al. [7], 26.08% of Belisário et al. [10] and 5.08% of Flanagan et al. [9], while homozygosity for the *GOLGB1* rs3732410_G allele was not found in our patients as in Flanagan's studies but contrary to 1.67% in Martella's report. Homozygotes for the *PON1* rs662_C allele occurred in a frequency of 10,3% in our study, whereas Martella et al. [7] and Flanagan et al. [9] reported 45% and 13.7%, respectively. Of the three variants, only *ENPP1* rs1044498 AA and AC genotypes showed a positive association with stroke risk. Notably, rs1044498_A is the minor allele, in our study group, while the variant allele (C) is the most frequent, which is in line with the reference population (African Yoruba) and contrary to what is described for the other reference populations. This may reflect a negative selection for the less advantageous allele - rs1044498_A in these populations. As for the *PON1* rs662_C variant, albeit no association with stroke or global CV was apparent, we observed a positive association with high platelet levels, indicating a potential impact on hemostasis and inflammation.

The only consensual modifiers of SCD disease severity, so far, have been the persistence of HbF beyond infancy and the presence of -$\alpha^{3.7kb}$–thal deletion. The co-inheritance of -$\alpha^{3.7kb}$–thal and homozygous HbS mutation has been associated with an overall ameliorating effect on anemia, particularly a protective effect against stroke in children [28]. We did not find any direct relationship between the presence of the -$\alpha^{3.7kb}$–thal deletion and stroke, although we did find that patients with α–thal showed a higher RBC count, lower MCV and MCH, consistent with previous reports [29,30] of ameliorating anemia factors. Other authors have also reported an absence of association between -$\alpha^{3.7kb}$–thal presence and stroke protection [31,32]. Despite the small sample size in our study, we cannot exclude that population heterogeneity or other specific population characteristics may contribute for the lack of association observed. Additionally, the unexpected association with increased neutrophil count might lower the above mentioned potentially favorable effect by indicating a proinflammatory role. The latter was also found in subjects with the Bantu haplotype while the Senegal haplotype seems to have the opposite effect, ameliorating inflammation and the hematological indices. Nevertheless, in our study, no *HBB* cluster haplotypes were found to be associated with CV.

5. Conclusion

Although the sample size in our study limits extrapolation to the general SCA pediatric population, our results seem to reinforce the importance of genetic modulators in the pathophysiology of cerebral vasculopathy and provide clues for the discovery of novel targets for SCA therapy. Our findings lead us to suggest that a comprehensive biomarker panel that includes biochemical, hematological, imaging as well as genetic data may be very important for CV prediction, and prevention. Even though the patients we studied are subjected to environmental, both physical and social, factors different from those to which the populations their parents originated from have been exposed, the genetic modifiers described in our study, namely *VCAM1* haplotype 7 and rs1409419, may provide further tools for CV prevention in SCA. Functional studies are of the utmost importance, not only for confirmation purposes, but also to assess the mechanisms by which the phenotype modulation may occur, and the potential use of these variants as novel genetic biomarkers of disease prognosis.

Author statement

Due to the sensitive nature of the question asked in this study, survey respondents were assured raw data would remain confidential and would not be shared.

CRediT authorship contribution statement

Marisa Silva: Methodology, Software, Formal analysis, Investigation, Data curation, Writing - original draft.**Sofia Vargas:**Methodology, Software, Formal analysis, Investigation,

Data curation.**Andreia Coelho**:Methodology, Software, Formal analysis, Investigation, Data curation.**Emanuel Ferreira**:Methodology, Software, Investigation.**Joana Mendonça**:Methodology, Software.**Luís Vieira**:Methodology, Software, Funding acquisition.**Raquel Maia**:Resources, Writing - review & editing. **Alexandra Dias**:Resources, Writing - review & editing.**Teresa Ferreira**:Resources, Writing - review & editing.**Anabela Morais**:Resources.**Isabel Mota Soares**:Resources, Writing - review & editing.**João Lavinha**:Funding acquisition, Writing - review & editing.**Rita Silva**:Resources, Writing - review & editing.**Paula Kjöllerström**:Resources, Writing - review & editing.**Paula Faustino**:Project administration, Funding acquisition, Methodology, Supervision, Writing - review & editing.

Declaration of competing interest

The authors have no competing interests.

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Appendix A. Supplementary data

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